

Coupling Growth Arrest and Adipocyte Differentiation

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The complete differentiation program of preadipose cells can be divided into early and late events. The expression of early markers takes place at growth arrest (G₁/S boundary), whereas that of late markers, leading to terminal differentiation, takes place after a limited number of mitoses of early marker-containing cells. Only terminal differentiation requires the presence of growth hormone and triiodothyronine and results in the formation of triacylglycerol-filled, nondividing cells. The events of adipose cell differentiation which take place *in vitro* allow a better understanding of the development of adipose tissue *in vivo*.

Introduction

The relationships between proliferation and differentiation of preadipose cells have not been delineated as extensively as those in other cell types. Studies on the coupling between growth arrest, or growth resumption, and adipocyte differentiation have been made possible in the last decade by the establishment of preadipocyte cell lines. 3T3-L1 and 3T3-F442A lines, subclones of 3T3 cells, were the first clonal lines described by Green and Kehinde (1,2). Both lines were established from total mouse embryo. The Ob17 clonal line (and derived subclones Ob1771 and Ob1754) and HGFu clonal line have been established in our laboratory from the epididymal fat pad of genetically obese (3) and genetically nonobese mice (4), respectively. Despite extensive chromosomal rearrangements taking place during the establishment of mouse clonal lines, nondifferentiated 3T3-F442A (5) or Ob17 cells (6), when injected into nude mice, are able to differentiate into fat pads containing fully mature fat cells. These results demonstrate that preadipocyte cells, as defined by *in vitro* studies, can be safely considered as true precursor cells of adipocytes.

Main Features of the Adipose Conversion Process

The process of adipose conversion of preadipocyte cells is phenotypically similar with respect to the various clonal lines. After the cells reach confluence, the cell shape changes. Fibroblastlike cells become round, enlarge, and will later accumulate lipid droplets in their cytoplasm. In serum-supplemented medium, the adipose conversion of precursor cells of established cell lines or that of precursor cells in primary culture, after their isolation from adipose tissue, does involve a limited proportion of cells. Typically, precursor cells from clonal lines will differentiate as colonies of fat cells separated from each other by cells insensitive to adipose conversion (Fig. 1). Insusceptible cells have clearly the same potentiality to differentiate because a) after their separation from differentiated cells, inoculation, and growth to confluence, a differentiation process into adipose cells of similar magnitude is observed and b) in serum-free, chemically defined medium, all cells can become differentiated (7,8).

The adipose conversion of Ob17 cells in serum-supplemented medium requires various hormones. Using bovine sera depleted of any of these three hormones, the acquisition of a fatty phenotype takes place in the presence of growth hormone (GH), triiodothyronine (T₃), and insulin (Fig. 1). When present within a physiological range of concentrations, both T₃ (9) and GH (10,11) act as obligatory hormones and are required on a long-term basis for terminal differentiation leading to triacylglycerol accumulation (9-11).

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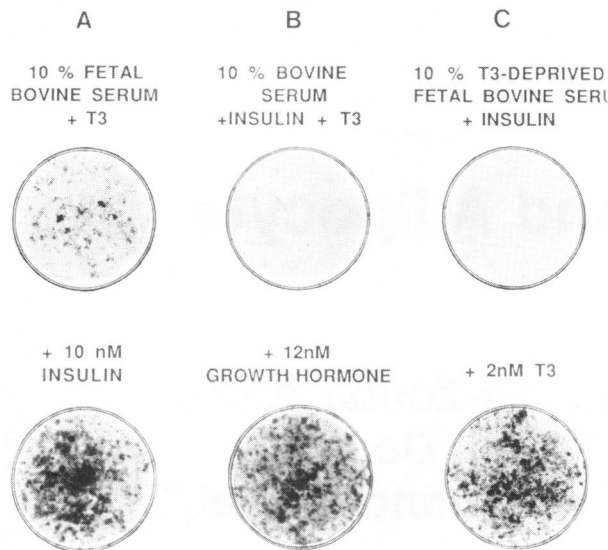


FIGURE 1. Hormonal requirements for the differentiation of preadipose cells. (A) Insulin supplementation; (B) GH supplementation; (C) T_3 supplementation. Ob17 cells are grown to confluence in a Dulbecco's modified Eagle's medium containing (A) 10% fetal bovine serum, (B) 10% bovine serum, or (C) 10% T_3 -depleted FBS. At confluence, culture media (changed every other day) are further supplemented with (A) 10 nM insulin, (B) 12 nM GH, or (C) 2 nM T_3 . Twelve days later, fat cell clusters are stained with Oil Red O. The pictures show clearly the presence of fat cell clusters in the absence of insulin (modulating hormone), but no cluster is visible with the naked eye or with the microscope in the absence of added GH or T_3 (obligatory hormones).

Insulin behaves as a mere modulator in the expression of the differentiation program (12–15). When insulin is present, the number of cell clusters remains unchanged, whereas the mitogenic effect of insulin at high concentrations slightly increases the average cell number per cluster but also increases the number of insusceptible cells (11). Clearly, insulin enhances the expression of various differentiation-specific phenotypes, including neutral lipid accumulation. As a consequence, the number of fat cell clusters visible with the naked eye after lipid staining is increased (Fig. 1). It is noticeable that in Ob17 cells the binding parameters of growth hormone and insulin to cell surface receptors (11,16) and those of T_3 to nuclear receptors (17,18) are in good agreement with the concentrations actually required for terminal differentiation.

The occurrence of early and late events in the adipose conversion process is supported by various lines of evidence and is illustrated both at the protein level (Fig. 2A) and at the mRNA level (Fig. 2B). Just after confluence, at a period of time during which most cells cease to undergo mitoses, lipoprotein lipase (LPL) appears rapidly, whereas glycerophosphate dehydrogenase (GPDH) is not yet expressed and appears later. The maximal expression of GPDH is significantly delayed as compared to that of LPL (Fig. 2). Under conditions where both the time course of differentiation is shortened in the presence of a cAMP-elevating agent and the differentiation-specific mRNAs are quantitated, the same order of emergence

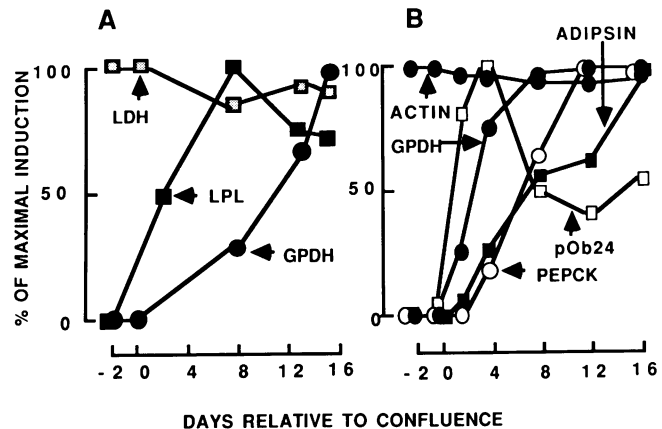


FIGURE 2. Early and late events during differentiation of preadipose cells. Ob17 cells are grown in DME medium containing 10% fetal bovine serum and further supplemented at confluence with 2 nM T_3 and 17 nM insulin. (A) Emergence of LPL and GPDH activities. Lactate dehydrogenase (LDH) activity does not change significantly with differentiation and has been used as internal control. (B) Emergence of pOb24 mRNA and various mRNAs encoding for GPDH, PEPCK, adipsin, and β -actin. Like β -actin mRNA, LDH mRNA content remains similar at each time (not shown).

still holds. Among early markers, the pOb24 mRNA (6 kb) (19) appears rapidly (in a parallel way to the two LPL mRNA species of 3.3 and 3.7 kb) (Fig. 3) and decreases afterwards, whereas emerging in the following order are the mRNAs encoding for GPDH (20), phosphoenolpyruvate carboxykinase (PEPCK) (21) and adipsin (22). The emergence of GPDH is critical for neutral lipid accumulation (23). The role of PEPCK in adipose tissue remains unclear, but it is known that adipsin is a protein homologue of a serine protease that is secreted and detected in the circulation (24,25). Early and late events in the adipose conversion process can also be distinguished by a) the isolation of LPL-containing cells devoided of triglycerides (23); b) the isolation of a polyamine-dependent variant of Ob17 cells (Ob1754 clonal line) able to express only early markers unless putrescine is added (26); and c) the demonstration that in serum-free, chemically defined medium, LPL-containing cells are present, able to undergo terminal differentiation, and able to accumulate lipids in the presence of serum adipogenic factors that are different from GH and T_3 and appear to be, at least in part, represented by arachidonic acid (27).

Time-course studies by Pairault and Green have indicated that, during the adipose conversion of 3T3-F442A cells maintained in suspension culture, DNA synthesis precedes a dramatic increase in GPDH activity (28). In the Ob17 clonal cell line, both DNA synthesis and post-confluent mitoses are only significant in cells that ultimately convert into triglyceride-filled cells (12) and that are also the cells that contain GPDH. These observations are consistent with a few mitoses of early marker-containing cells taking place before or during terminal differentiation. The existence of such a limited proliferation of differentiating cells has been shown directly by the ability of LPL-containing Ob1754 cells, when exposed to

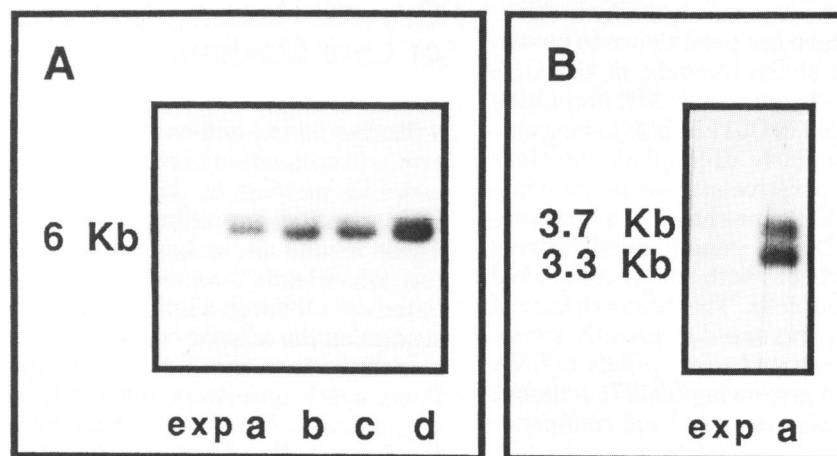


FIGURE 3. Emergence in growth-arrested preadipocyte cells of early differentiation-specific markers. Growth arrest is initiated by addition of 5 mM thymidine to Ob17 cells actively growing in DME medium supplemented with 10% fetal bovine serum or 10% bovine serum in the absence of any hormonal supplementation; similar results have been obtained using cells exposed to a double-thymidine block (18). Growth arrest can also be initiated by adding 3 μ M aphidicolin. Control experiments show that more than 90% of the cells are synchronized and are blocked at the entry of the S stage of the cell cycle. Twenty-four hours after thymidine or aphidicolin addition, or 24 hr after Ca^{2+} or serum deprivation, poly(A)-containing RNAs are prepared and analyzed for the presence of pOb24 mRNA (A) and LPL mRNAs (B). The symbols are: exp, exponentially growing cells; a, thymidine block; b, aphidicolin block; c, Ca^{2+} deprivation; d, serum deprivation.

putrescine, to synthesize DNA, to undergo at least one round of mitosis, to express GPDH, and to accumulate neutral lipids (26). This sequence of events delineated *in vitro* is in agreement with various *in vivo* observations showing that a) cells of the stromal-vascular fraction of adipose tissue from adult mice, i.e., cells still having no significant lipid accumulation, do contain both the two LPL mRNA species and a high concentration of pOb24 mRNA. In contrast, the GPDH mRNA is present at a low concentration, whereas the adipsin mRNA is barely detectable; b) in the rat, after pulse labeling with [^3H]thymidine, the labeling indices of cells from the subcutaneous adipose tissue are highest in partially differentiated cells (esterase positive) containing no lipid droplets (29); and c) in mice, the decrease in the labeling index of GPDH-negative cells immediately precedes the rise of this enzyme detected subsequently in all triacylglycerol-filled cells (30).

At this point, it appears at first sight that the expression of early markers is coupled to growth arrest, whereas that of late markers is linked to a limited growth resumption. Both events have been examined more precisely and are discussed in the following sections.

Growth Arrest and Expression of Early Markers

The necessity of a growth arrest for adipose cell differentiation is clearly illustrated when Ob17 cells are transformed by the middle-T-only gene of polyoma virus. Among the different clones obtained, there is an inverse relationship in culture between their potentiality to overproliferate at low serum and their potentiality to convert into adipose cells (31). In addition, when cells of a given

clone become overexposed by steadily increasing the concentration of a mitogen such as $\text{PGF}_{2\alpha}$, the proportion of adipose-converted cells is decreased (32).

Therefore, it has been reasoned that whether the expression of early markers were related to growth arrest, exponentially growing cells should be able after growth arrest, to express early, differentiation-specific markers. This prediction seems fulfilled, as illustrated in Figure 3. After a single- or double-thymidine block of actively growing cells, i.e., in the absence of intercellular contacts, the emergence of pOb24 mRNA and LPL mRNAs in both Ob17 and 3T3-F442A cells is rapid; it is confined to early markers because GPDH mRNA remains undetectable. The expression of pOb24 mRNA has been studied in some detail, as it appears to be absent both in growing and growth-arrested 3T3-C2 cells, a clonal line showing a low frequency of adipose conversion (2,19), and present in adult mice in the adipose precursor cells of adipose tissue but undetectable in liver, kidney, heart, spleen, skeletal muscle, and brain. The expression of pOb24 mRNA can be considered as a marker of cell commitment, which can also be induced by other blocks; it takes place in a similar way in the presence of aphidicolin, as well as after Ca^{2+} or serum deprivation (Fig. 3). In each case, block removal is followed within a few minutes by DNA synthesis, even when the block has occurred by serum deprivation.

These results strongly suggest that the cells that have been growth arrested under these various culture conditions are blocked near or at the G_1/S stage rather than at an early G_1 stage of the cell cycle. This interpretation is supported by the fact that, in contrast to Ob17 and 3T3-F442A cells, 8 hr are needed for 3T3-C2 cells to resume DNA synthesis after serum deprivation. It is also

supported by the fact that the expression of dihydrofolate reductase (DHFR) gene, which has been shown to be constitutive with a brief and sharp increase at the G₁/S boundary in metotrexate-resistant mouse 3T6 fibroblasts (33,34), is also increased 4-fold in Ob17 cells following thymidine block (Fig. 4), and similarly after aphidicolin block or Ca²⁺ deprivation. Comparative studies of the transcription of pOb24 and DHFR genes have thus been performed. As expected, the DHFR gene is constitutively transcribed in growing Ob17 cells with a more than 4-fold increase in growth-arrested cells. The transcription of pOb24 gene is dramatically increased at growth arrest. A low transcriptional activity and a low pOb24 mRNA content, if any, are observed in growing Ob1771 cells, but this is partly, if not totally due to some local confluence (Fig. 4).

It is unclear at the present time whether the expression of pOb24 mRNA is critically required for the subsequent events of terminal differentiation and whether it is not a mere consequence of various events occurring at growth arrest. Although a possible answer will only be given by sequence studies of the pOb24 cDNA and functional studies of the corresponding protein, we would favor the first alternative, as resumption of active and continuous growth after block removal leads within a couple of hours to a dramatic decrease of the transcription rate of pOb24 gene and to a cell decommitment, leading in turn to a complete dedifferentiation (19) and under all the various conditions favoring terminal differentiation and lipid accumulation, a prior expression of pOb24 mRNA has been consistently detected.

It is of interest to observe that the expression of pOb24 mRNA does not require and is not regulated by insulin, GH, and T₃ (Fig. 5), whereas, GH and T₃, which are needed for terminal differentiation (Fig. 1), are actually required for the expression of GPDH mRNA (and for that of the protein) (10).

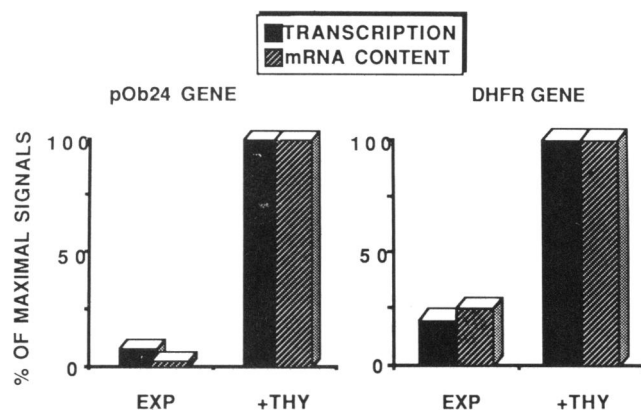


FIGURE 4. Regulation of pOb24 and DHFR gene expression in growth-arrested Ob1771 cells. The results reported here have been obtained with the pBr 322 DNA plasmid as control, in order to exclude any unspecific hybridization. Note that both the emergence of DHFR mRNA and the transcription of the DHFR gene do take place significantly in growing cells (33,34).

Growth Resumption and Expression of Late Markers

Studies on the adipose conversion of 3T3-F442A cells grown in suspension have shown that DNA synthesis precedes an increase in GPDH activity (28). Inhibition of DNA synthesis prevents the formation of fat cell clusters. These results are in favor of an amplification phenomenon, which leads to an increase in the number of differentiated cells through a limited number of mitoses. Further studies on the adipose conversion of Ob17 cells grown as monolayer have shown that DNA-synthesizing cells are those which ultimately convert to adipose cells and in which cluster formation is indeed abolished by blocking DNA synthesis of early confluent (but not of late confluent) cells (13). Both sets of observations suggest that the amplification process, which is linked to growth resumption, is limited both in magnitude and duration, i.e., to a short and critical period of time after confluence.

Various lines of evidence directly support this hypothesis. First, LPL-containing Ob17 cells are able to synthesize DNA (23). Second, it is possible to obtain a full expression of LPL following growth arrest by thymidine of actively growing Ob17 cells (Fig. 3). At that point, 70 to 90% of the cells express LPL [detected by immunofluorescence staining according to Vannier et al. (35)]. Growth-arrested cells can then be dissociated and reinoculated at high density. As shown in Figure 6, DNA synthesis resumes immediately after block removal, and the cell number increases 1.6-fold within the first 24 hr. On day 6, the cell number has increased 2.5-fold, the GPDH activity emerges at that time and increases onwards. Third, in the polyamine-dependent Ob1754 clonal line, the appearance of LPL precedes DNA synthesis and postconfluent mitoses (one cell doubling observed), which are both putrescine-dependent and which take place before the appearance of the bulk of GPDH activity (Fig. 7). These results, with respect to growth arrest and expression of early markers on one hand and limited growth resumption and expression of late markers on the other hand, could be interpreted as shown in Figure 8.

We assume that early mRNA and protein markers are synthesized during the first G₁ stage of the cell cycle (G_{1A}). Precursor cells containing these markers, i.e., pOb24 and LPL mRNAs, are indeed present *in vivo* and could represent dormant cells likely arrested at this stage. Early marker-containing cells would then divide at least once, would express late markers during the G₁ stage of a second cell cycle (G_{1B}), and would ultimately express GPDH, accumulate triacylglycerol, and cease cell division. Terminal differentiation both *in vitro* and *in vivo* could only take place after growth resumption in the presence of the appropriate hormonal milieu.

In vitro, the irreversible entry of early marker-containing cells into the process of terminal differentiation requires not only hormones but also a situation favoring cell-to-cell proximity. Such proximity would be, of course, maximal at a density near or at saturation, and we have some evidence that some prostaglandins synthesized

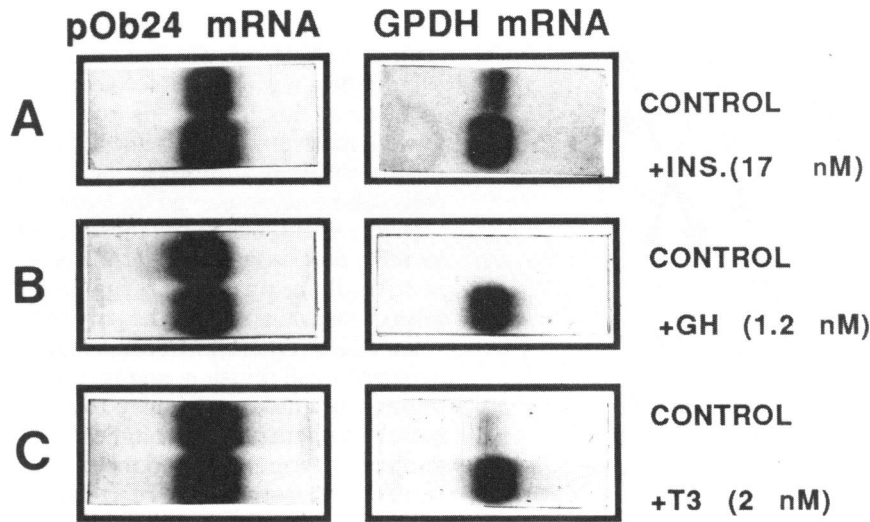


FIGURE 5. Differential hormonal requirement for the emergence of pOb24 and GPDH mRNAs. Ob1771 cells are grown in DME medium supplemented with (A) 10% fetal bovine serum, (B) 10% bovine serum, or (C) 10% T₃-depleted fetal bovine serum. As indicated, at confluence, culture media are further supplemented with (A) 17 nM insulin, (B) 1.2 nM GH, or (C) 2 nM T₃. Poly(A)-containing RNAs are prepared and analyzed for the presence of pOb24 and GPDH mRNAs at (A) day 11, (B) day 14, and (C) day 12 postconfluence.

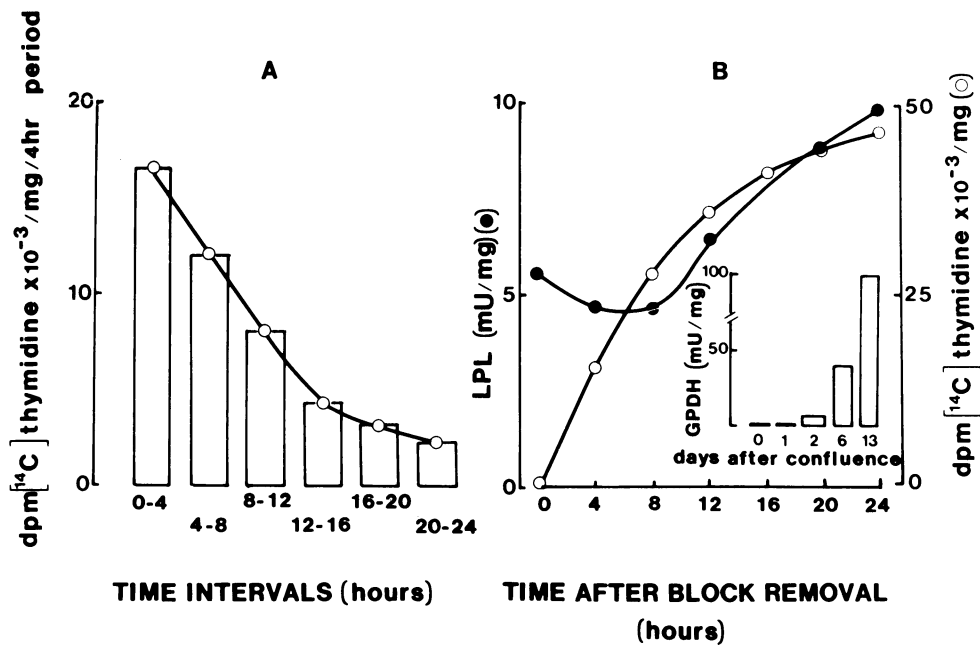


FIGURE 6. LPL and GPDH activities in growth-arrested Ob1771 cells after block removal. Growth-arrested Ob1771 cells (double-thymidine block) are reinoculated at high density (35×10^3 cells/cm²) in culture medium supplemented with 5 mM thymidine. After 4 hr, which are required for the cell attachment (plating efficiency 65–80%), cells are exposed to fresh DME medium containing 10% fetal bovine serum, 2 nM T₃, and 17 nM insulin. (A) 2-¹⁴C-thymidine incorporation into DNA when cells are pulsed for 4 hr between time zero (defined as block removal) and 24 hr; (B) 2-¹⁴C-thymidine incorporation into DNA when cells are continuously exposed to the labeled precursor from time zero. LPL and GPDH activities are determined after block removal as indicated. The cell number is increased 1.6-fold and 2.5-fold 1 day and 6 days following block removal, respectively. Reproduced from Amri et al. (19).

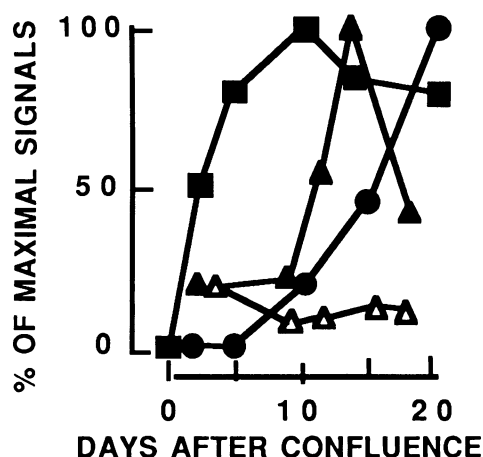


FIGURE 7. Growth resumption and expression of GPDH in Ob1754 cells. Confluent Ob1754 cells are chronically exposed after confluence to the same differentiation medium used in Fig. 6 supplemented (■, ▲, ●) or not (△) with 100 μ M putrescine and 10 μ M methylglyoxal bis(guanyl)hydrazone (26). DNA synthesis (▲, △) is determined from day 5 to day 20 by 3 H-thymidine incorporation into DNA for 48 hr into trichloroacetic acid-precipitable material. LPL (■) and GPDH (●) activities are determined at the days indicated. Cell enumeration at day 22 shows above a 2-fold increase in putrescine-treated cells (as compared to day 0) and a 1.2-fold increase in untreated cells.

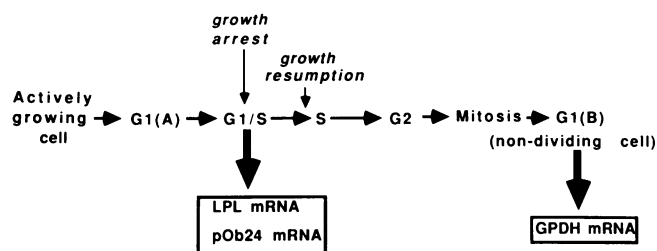


FIGURE 8. Coupling between the various stages of the cell cycle and adipose cell differentiation.

by differentiating Ob17 cells might play as diffusible factors a critical autocrine/paracrine role.

With respect to the situation of adipose tissue development *in vivo*, two points deserve comments. First, the proximity of differentiating cells has been unambiguously demonstrated in various studies (30,36,37). Second, both *in vitro* and *in vivo*, one cannot exclude that either cells that are devoided of triglycerides, i.e., not having yet ex-

pressed GPDH, or cells that are not yet fully mature cells, can divide a few times before complete cessation growth. In favor of this hypothesis are recent experiments performed in serum-free, chemically defined medium that indicate terminal differentiation of rat adipose precursor cells can be accompanied by more than one cell doubling.

In any event, one of the most important questions both *in vitro* and *in vivo* is whether at least one cell division is critically required for terminal differentiation to take place. The availability of the polyamine-dependent Ob1754 cells, which require putrescine supplementation for DNA synthesis, cell division, and terminal differentiation (26), offers a unique opportunity to shed some light on that point. The data in Table 1 indicate that, in putrescine, T_3 , and insulin-supplemented medium containing bovine serum, the expression of GPDH mRNA requires the presence of GH and at least one mitosis; this mRNA is undetectable in Ob1754 cells in the presence of GH but in the absence of the mitogenic stimulus. A low but significant accumulation of GPDH mRNA in the presence of a mitogenic stimulus despite the absence of added GH has been observed; this is likely due to the low but significant endogenous content of GH in bovine serum. It is of interest to compare the expression of GPDH mRNA with those of the insulinlike growth factor I (IGF-I) mRNA and adipsin mRNA, as their expression in Ob1771 cells requires the presence of GH in both cases, but time course studies indicate that the emergence and the accumulation of IGF-I mRNA takes place before growth resumption (38). In contrast, the accumulation of adipsin mRNA takes place after cell division, i.e., during very late terminal differentiation (21). Table 1 shows clearly the dependence upon GH for the expression of these two differentiation-specific mRNA markers. It also shows that, as anticipated, no mitogenic stimulus is required in Ob1754 cells for the expression of IGF-I mRNAs (7.5, 1.5, and 0.8 kb) (38) and possibly of IGF-I. However, in contrast to that of GPDH mRNA, the expression of adipsin mRNA (1 kb) (22) does not require cell division. In other words, only the emergence of GPDH mRNA, that of the corresponding enzyme, and, ultimately, the accumulation of neutral lipids, are critically coupled to this limited growth resumption, providing that GH and T_3 are present. If both hormones are absent, such mitosis still occurs but becomes abortive with respect to terminal cell differentiation. This observation *in vitro* provides the critical clue to understanding the terminal differentiation process of fat cells from precursor cells *in vivo* and suggests the ex-

Table 1. Relationships between postconfluent mitoses, growth hormone requirement, and expression of differentiation-specific markers.

Addition at confluence	Postconfluent mitoses	mRNA, % ^a				
		pOb24	LPL	IGF-I	GPDH	Adipsin
None	—	55	65	0	0	0
MGBG, 10 μ M/putrescine, 100 μ M	+	100	95	0	3	0
GH, 1.2 nM	—	60	80	90	0	105
MGBG 10 μ M/putrescine, 100 μ M/GH, 1.2 nM	+	100	100	100	100	100

^aThe results are expressed in percent by taking as 100% the signal obtained for each specific mRNA in confluent Ob1754 cells exposed for 15 days as indicated (see Fig. 7 for details).

istence of a delicate balance between growth arrest leading to dormant, partly differentiated cells and a situation whereby these cells resume limited proliferation and differentiate irreversibly into mature fat cells.

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